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Effects of Proteolytic Digestion on the Control Mechanism of Ciliary Orientation in Ciliated Sheets from *Paramecium*

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ABSTRACT—Effects of proteolytic digestion on the ciliary response to Ca^{2+} and cyclic nucleotides in ciliated cortical sheets from *Paramecium* were examined. Ciliary orientation toward 5 o'clock (with the anterior of the cell defined as 12 o'clock) in response not only to lowering Ca^{2+} concentration, but also to raising cyclic nucleotides concentrations, was lost by trypsin digestion. Differential sensitivity of cilia, depending on the location of the cell surface to cyclic nucleotides, was also lost by trypsin digestion. The cilia reoriented toward 12 o'clock, which was the ciliary orientation in response to Ca^{2+} above 1 μM before trypsin digestion. Ciliary axonemes lost a large number of outer dynein arms after trypsin digestion. This suggests that the outer dynein arm may contribute to controlling the ciliary beating direction in response to intracellular Ca^{2+} and cyclic nucleotides.

INTRODUCTION

The locomotor behavior of *Paramecium* depends on the ciliary beating direction which is controlled by intracellular Ca^{2+} concentration [3, 7–9] and presumably by cyclic nucleotides concentration [1, 4, 6, 11]. We observed the top view of ciliary orientation controlled by Ca^{2+} and cyclic nucleotides in ciliated cortical sheets from demembranated cell model of *Paramecium,* and found that cyclic nucleotides competed with Ca^{2+} in determining the ciliary orientation [14]. Also, it was revealed that the ciliary sensitivity to cyclic nucleotides depended on the location of the cell surface [14].

We reported that both the change of ciliary orientation and the stable position in response to lowering the Ca^{2+} concentration disappeared by trypsin digestion using Triton-glycerol-extracted *Paramecium* [13]. This seemed to occur as a result of selective digestion of the Ca^{2+}-dependent controlling mechanism of ciliary beating. It is important to examine the changes that proteolytic digestion induces in ciliary orientation on the cortial sheets of *Paramecium,* and the structural changes in ciliary axonemes after proteolytic digestion.

We report here observations of ciliary orientations on cortical sheets of Triton-glycerol-extracted *Paramecium* undergoing proteolytic digestion. Tryptic digestion removed the cyclic nucleotide sensitivity, as well as Ca^{2+} sensitivity, in the ciliary reorientation response on the cortical sheets. After trypsin digestion, the cilia pointed toward 12 o'clock irrespective of cyclic nucleotides and Ca^{2+} concentrations. Electron micrographs showed that a large number of outer dynein arms disappeared after trypsin digestion. The results suggest that the mechanism which controls ciliary orientation, depending on intracellular Ca^{2+} and cyclic nuceotides, may be closely related to the outer dynein arms.

MATERIALS AND METHODS

Paramecium caudatum (stock G3) was cultured in a hay infusion. Cells were grown to late-logarithmic phase at 25°C.

Preparation of ciliated sheets of permeabilized

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cells and reactivation of cilia were essentially the same as the preceding paper [14]. Concentrated and washed cells were extracted with an extraction medium containing 0.1% Triton X-100, 20 mM KCl, 10 mM EDTA and 10 mM Tris-maleate buffer (pH 7.0) for 5 min in an ice bath. The extracted specimens were then washed three times with an ice-cold washing medium which consisted of 50 mM KCl, 2 mM EDTA and 10 mM Tris-maleate buffer (pH 7.0). At that time they were pipetted several times through a glass pipette with a small inside tip diameter to tear or to give a nick to the cell cortex. Then the models were suspended and equilibrated in an ice-cold glycerol-KCl solution which consisted of 30% glycerol, 50 mM KCl and 10 mM Tris-maleate buffer (pH 7.0) prior to experimentation.

A simple perfusion chamber was prepared by placing the sample between a slide and a coverslip. The slide and the coverslip were separated by a thin layer of vaseline applied to two opposite edges of the coveslip. To observe the reactivation of cilia on the sheet of cell cortex, 20 μl of the sample was placed on a slide glass, and a coverslip with vaseline was placed gently on the sample. Solutions were then perfused through the narrow opening at one of the edges of the coverslip while the excess fluid was drained from the opposite end with small pieces of filter paper. During the first perfusion using a reference glycerol-KCl solution, some torn cell cortex adhered flat to the glass surface. The cortical sheets were then perfused successively with reactivation solutions. All the reactivation solutions contained 1 mM ATP, 1 mM $MgCl_2$, 30% glycerol, 50 mM KCl and 10 mM Tris-maleate bufer (pH 7.0) as well as the component(s) noted in the results. Free Ca^{2+} concentration in the reactivation solution was controlled by a Ca-EGTA bufer with 1 mM EGTA [12, 15]. The reactivation was carried out at 22–25°C. The movements of the cilia were observed under a dark field microscope, equipped with a 100 W mercury light source, a heat filter and a green filter, and recorded on video tape using a National WV-1550 TV camera. Pointing directions of cilia which were observed from above were indicated by using a clock face with a definition of the anterior end of the cells as 12 o'clock. The anterioposterior axis of cortical sheets were determined by anterioposterior lines of cilia. Ciliary orientation of cilia were measured as angles to an anerioposterior axis and then converted to an analogue clock face expression using each cilium on the cell surface except for the oral groove. With the definition that the surface area of the anatomical left-hand side is the left-hand field of the cortical sheets, we used the cilia on the left third and the right third for measuring the ciliary orientation of the left-hand field and the right-hand field, respectivley. In the measurement of ciliary orientation after trypsin digestion, we used the cilia on both the left- and right-hand field.

Prepared Triton-glycerol-extracted whole cell models were suspended in a reactivation solution containing 100 μg/ml trypsin (from bovine pancreas, Boehringer, 110 units/mg) or elastase (from porcine pancreas, Sigma type IV, 120 units/mg) in a test tube to make the cellular protein concentration approximately 1 mg/ml. A minute amount of the suspension was placed on a slide glass and the ciliary response to digestion was observed under the dark field microscope. Five minutes after mixing, soybean trypsin inhibitor was added to make the final concentration of 1.0 mg/ml. The samples were fixed with 2% glutaraldehyde in 50 mM sodium cacodylate (pH 7.4) for 1 hr then post-fixed for 1 hr with 1% OsO_4 in the same buffer. They were dehydrated in an ascending series of ethanol to 100% and embedded in epoxy resin following the method of Nakamura [10]. Sections were cut on a Porter-Blum MT-1 ultramicrotome and were stained with uranyl acetate and Reynold's lead citrate. A JEOL 100C electron microscope was used for the observations.

RESULTS

The cilia on the cortical sheets reoriented toward 12 o'clock after trypsin digestion irrespective of Ca^{2+} concentration (Fig. 1). When the Ca^{2+} concentration was low enough to produce ciliary orientation toward 5 o'clock, which corresponded to normal ciliary beat, and consequent forward swimming [14], almost quiescent cilia pointing 5 o'clock began to rotate counterclockwise within a minute after perfusing a reactivation

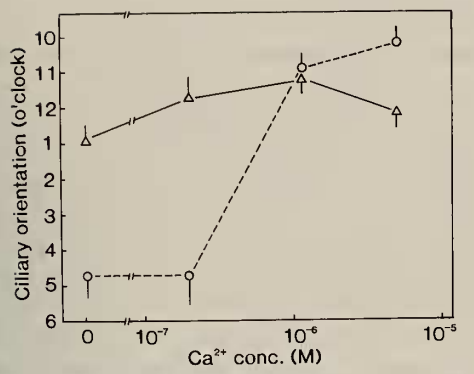

FIG. 1. Ciliary orientation in response to Ca^{2+} concentration after trypsin digestion. Circles and broken lines indicate the ciliary orientations before trypsin digestion. Triangles and solid lines indicate ciliary orientations 3–5 minutes after trypsin digestion. Ciliary orientation measured as angles to an anterioposterior axis and then converted to an analogue clock face expression as indicated in the ordinate. Measurements were performed about cilia on the dorsal area. The data was obtained from four individual preparations. The bars indicate standard deviations.

solution containing 100 μg/ml trypsin. Finally the cilia pointed toward 12 o'clock and stopped within a few minutes (Fig. 1). When the Ca^{2+} concentration was above 10^{-6} M, cilia which oriented toward 10–12 o'clock moving counterclockwise in a circular pattern stopped moving and finally pointed toward 12 o'clock within a few minutes after the perfusion of reactivation solution containing 100 μg/ml trypsin (Fig. 1).

As reported in the preceding paper [8], cAMP induces ciliary orientation toward 5 o'clock and cGMP induces ciliary orientation toward 3 o'clock, even in the presence of Ca^{2+} above 10^{-6} M which induces ciliary orientation toward 11–12 o'clock if cyclic nucleotides are not present. The cilia on the left-hand field of the cell are more sensitive to cyclic nucleotides than those on the right-hand field with the definition that the surface area of the anatomical left-hand side is the left-hand field of the cortical sheets. As shown in Figures 2 and 3, after perfusing a reactivation solution containing 100 μg/ml trypsin, the cilia oriented uniformly toward 12 o'clock irrespective of cyclic nucleotide concentrations and location of the cilia on the cell surface. Also in the presence of cyclic nucleotides without Ca^{2+}, trypsin digestion induces the ciliary orientation toward 12 o'clock (data not shown). Differential response of cilia to cyclic nucleotides in the left- and right-hand field of the cells disappeared by trypsin digestion (middle part of Figs. 2 and 3, and Fig. 4). Elastase which was thought to digest nexin [2], and V8 protease which produced a proteolytic effect different from trypsin on the 21S dynein from sea urchin sperm flagella [5], never produced such a change in ciliary orientation that was found to be induced by trypsin digestion in all cases previously described at concentrations of 100 μg/ml.

The cahnge of ciliary orientation after trypsin digestion in the absence of ATP was also tested to examine whether the changes of ciliary orientation by trypsin digestion were induced by active sliding or induced passively by structural changes of some

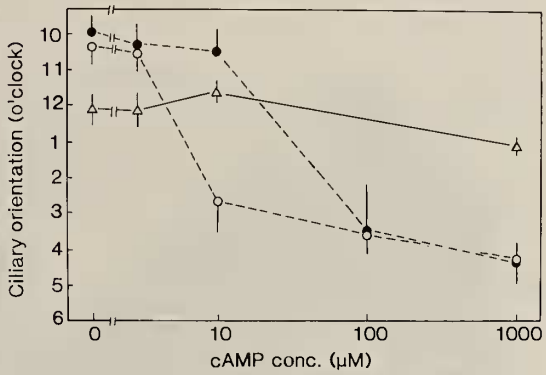

FIG. 2. Ciliary orientation in response to cAMP concentration after trypsin digestion. Ca^{2+} concentration is 5 μM throughout. Circles and broken lines indicate ciliary orientations before trypsin digestion. Open circles and closed circles indicate the ciliary orientations in the left-hand field and in the right-hand field, respectively. Triangles and solid lines indicate the ciliary orientations 3–5 minutes after trypsin digestion. Ciliary orientation of cilia were measured as angles to an anterioposterior axis and then converted to an analogue clock face expression as indicated in the ordinate. In the measurement of ciliary orientation before trypsin digestion, we used the left third and the right third of each cilium for measuring the ciliary orientation of the left-hand field and the right-hand field, respectively. In the measurement of ciliary orientation after trypsin digestion, we used the cilia on both the left- and right-hand field. The data was obtained from five individual preparations. The bars indicate standard deviations.

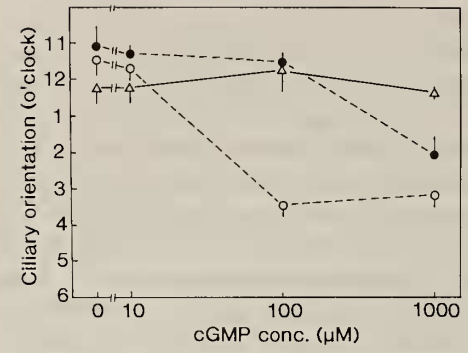

Fig. 3. Ciliary orientation in response to cGMP concentration after trypsin digestion. Ca^{2+} concentration is 1.8 μM throughout. Circles and broken lines indicate ciliary orientations before trypsin digestion. Open circles and closed circles indicate the ciliary orientations in the left-hand field and in the right-hand field, respectively. Triangles and solid lines indicate the ciliary orientations 3–5 minutes after trypsin digestion. Measurements of ciliary orientation were performed as explained in the legend of Figure 2. The data was obtained from four individual preparations. The bars indicate standard deviations.

axonemal components. As shown in Figure 5, the change of ciliary orientation from 5 to 12 o'clock induced by trypsin digestion required ATP. When the ciliated sheets were perfused with a reactivation solution containing EGTA to keep Ca^{2+} concentration under 10^{-7} M, the cilia pointed toward 5 o'clock (Fig. 5a). Following successive perfusions with a solution without ATP and a solution containing trypsin without ATP did not affect the ciliary orientation toward 5 o'clock. Eighty seconds after the trypsin perfusion, a solution containing a trypsin inhibitor without ATP was perfused. Then the reactivation solution con-

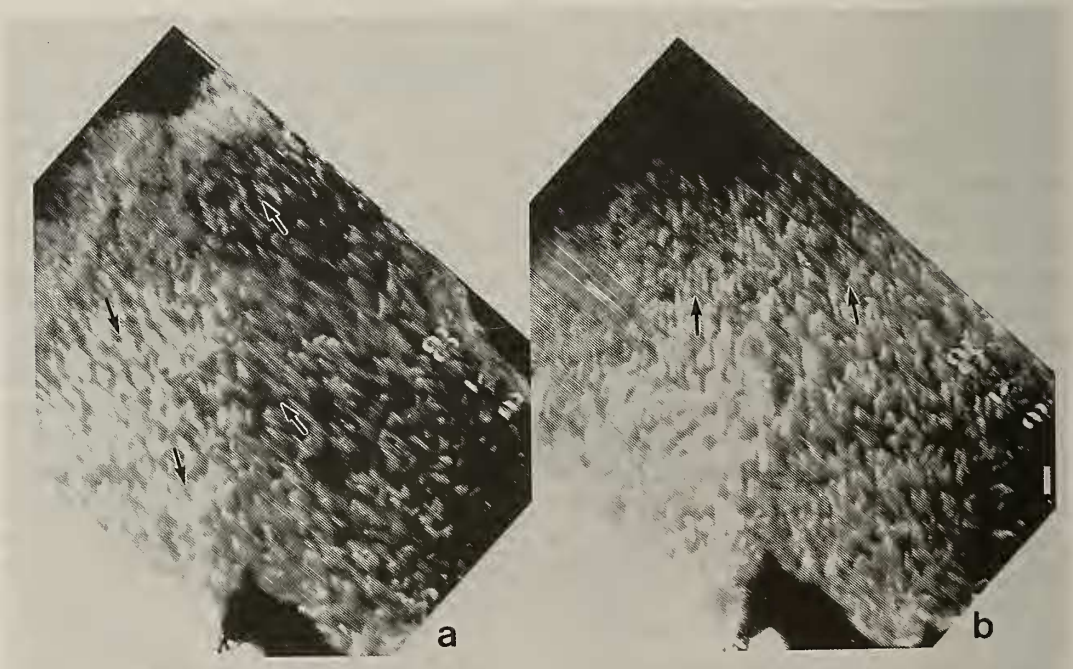

Fig. 4. Ciliary orientations before and after trypsin digestion. The photograph shows a dorsal view of the ciliated sheet. The cortex of a cell model tore along an oral groove. The top of each photograph is the anterior direction of the cell. The sheet was perfused successively with reactivation solutions containing 10 μM cAMP and 5 μM Ca^{2+} (a), and 100 μg/ml trypsin as well as 10 μM cAMP and 5 μM Ca^{2+} (b). Arrows indicate the pointing directions of cilia. Pointing direction of cilia on the left-hand field differed from that on the right-hand field in (a). In (a), as indicated by arrows, ciliary orientation on the left hand field was 5 o'lock, and on the right-hand field was 11 o'clock. On the other hand, ciliary orientation was 12 o'clock uniformly in the whole area of the cortical sheet after trypsin digestion (b). Bar, 10 μm.

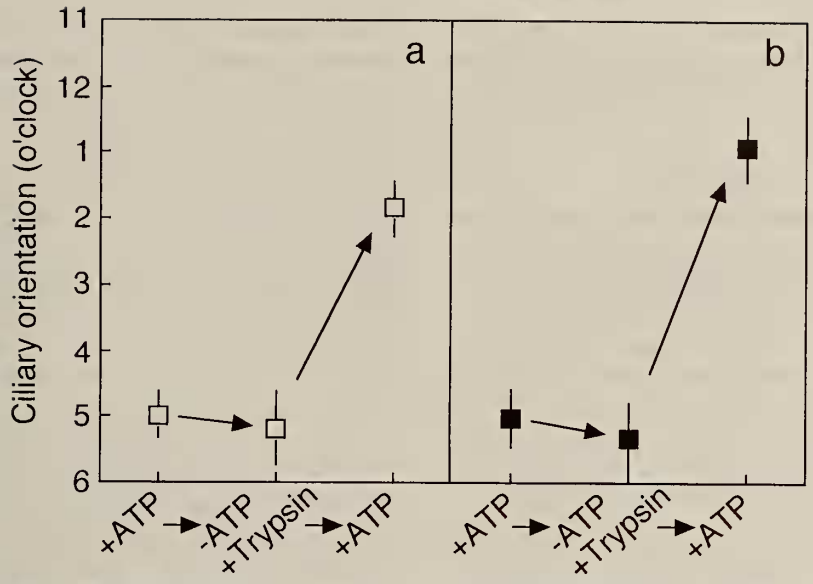

FIG. 5. The effect of trypsin digestion on the ciliary orientation in the absence of ATP. Cortical sheets were perfused successively with solutions fundamentally contained 1 mM $MgCl_2$, 30% glycerol, 50 mM KCl, Tris-maleate buffer (pH 7.0) as well as 1 mM EGTA (a) or 1 μM Ca^{2+} + 100 μM cAMP (b). +ATP indicated under abscissa means the solution contained 1 mM ATP. Trypsin concentration was 50 μg/ml. Cortical sheets were first perfused with solutions containing ATP. In both cases, ciliary orientations on the cortical sheets were not changed by subsequent trypsin digestion in the absence of ATP. After stopping the digestion by the addition of trypsin inhibitor, the solution containing ATP was perfused. The perfusion elicited the change of ciliary orientation from 5 toward 12 o'clock. Trypsin digestion time was 80 sec in (a) and 20 sec in (b). The data presented is a typical example of one of the many series of successive perfusions examined. The bars indicate standard deviations.

taining ATP was perfused. Immediately after the perfusion, the cilia changed their orientation from 5 o'clock to 12 o'clock and never returned toward 5 o'clock. The ciliary orientation toward 5 o'clock induced by cAMP also did not change the orientation by trypsin digestion in the absence of ATP (Fig. 5b). Thus, the change of ciliary orientation from 5 to 12 o'clock after trypsin digestion required ATP, and the trypsin digestion removed the ability to change and to keep the ciliary orientation toward 5 o'clock either in response to cyclic nucleotides or to lowering Ca^{2+} concentration.

We examined the effects of trypsin digestion on the axonemal structures by electron microscopy. The structure which underwent serious break down by trypsin digestion was outer dynein arms

FIG. 6. Cross-sections of the Triton-glycerol-extracted ciliary axonemes before proteolytic digestion (a) and after digestion by 100 μg/ml trypsin (b) and by 100 μg/ml elastase (c). bar, 100 nm.

(Fig. 6b). On the contrary, axonemes treated by elastase in the same condition as that of trypsin digestion kept their outer dynein arms (Fig. 6c).

DISCUSSION

Ciliary orientation on the cortical sheets corresponds to the ditection of the effective stroke of cilia [14]. The ciliary orientation toward 5 o'clock and 12 o'clock correspond to normal beating of cilia and ciliary reversal, respectively. The direction of the effective power stroke is essentially controlled by intracelular Ca^{2+} concentration [3, 7–9]. However, as we reported previously, "ciliary reversal" was induced by trypsin digestion [13] in Triton-glycerol-extracted whole cell model of *Paramecium* even if Ca^{2+} concentration was low enough to produce normal beating. In this paper, we confirmed that the ciliary orientation induced by trypsin digestion was in actual fact 12 o'clock using cortical sheets of Triton-glycerol-extracted *Paramecium* (Fig. 1). This indicates that the Ca^{2+}-dependent controlling mechanism of ciliary beating direction is digested at least in part by trypsin. The part affected by trypsin may be the component which is responsible for switching off the ciliary reversal mechanism when Ca^{2+} concentration is lowered.

The beating direction of cilia is also modulated by cyclic nucleotides [1, 6, 11, 14]. In the cortical sheets, ciliary orientation is controlled by cyclic nucleotides in the competing mode with Ca^{2+}. The cilia on the left-hand field of the cell surface were more sensitive to cyclic nucleotides than those on the right-hand field [14]. Tryptic digestion removed all these ciliary responses to cyclic nucleotides. As indicated in Figures 2–4, ciliary orientations towad 3–5 o'clock in response to cyclic nucleotides together with the differential sensitivity disappeared by trypsin digestion. This indicates that trypsin digestion destroys the component responsible for cyclic nucleotides dependent responses, as well as the Ca^{2+}-dependent component in the controlling mechanism of ciliary beating direction.

Ciliary orientation toward 5 o'clock, induced in the conditions in which Ca^{2+} concentration was low enough, or cAMP concentration was high enough to suppress the Ca^{2+} effect, remained the same after perfusing the solution without ATP. Subsequent perfusions with a solution containing trypsin without ATP, did not induce any change in ciliary orientation. However, the change of ciliary orientation from 5 to 12 o'clock was elicited by the addition of ATP (Fig. 5). This indicates that the change of ciliary orientation induced by trypsin digestion requires ATP and suggests that the change of ciliary orientation requires dynein-microtubul interaction at least to some extent. Thus, the trypsin digestion induced the inabilities to keep the ciliary orientation toward 5 o'clock and to change the ciliary orientation from 12 to 5 o'clock in response to either lowering Ca^{2+} concentration or to cyclic nucleotides.

It has been clarified that ciliary beating is controlled by Ca^{2+} [3, 7–9, 14] and cyclic nucleotides [1, 6, 11, 14]. However, the molecular mechanisms which transmit the signal of the second messengers to dynein-microtubule interaction as a motor system, as well as its localization and structural bases are scarcely known. After trypsin digestion, outer dynein arms disappeared conspicuously in ciliary axonemes (Fig. 6). On the contrary, outer dynein arms remained intact in the axonemes after the digestion by elastase which affected neither Ca^{2+} nor the cyclic nucleotides dependent mechanism as stated previously, whereas axonemes which detached from cell cortex and attached to the glass surface of the perfusion chamber exhibited sliding disintegration within a minute not only by trypsin but also by elastase. Trypsin has been used to produce limited digestion of isolated dynein [5], but how the trypsin affect outer dynein arms within axonemes is not yet known. Although we could not known the exact protein ratio of trypsin to ciliated sheets in perfusion chambers, high concentration of trypsin (above 20 μg/ml) was required to induce the ciliary response by trypsin digestion. This might imply that the quick attack by trypsin on the outside of axonemes is essential to remove selectively the Ca^{2+}-dependent and cyclic nucleotides-dependent controlling mechanism of ciliary orientation. The disappearance of the outer arms after tyrpsin digestion might suggests that outer dynein arms play a key role in Ca^{2+}-dependent

and also in the cyclic nucleotides dependent controlling mechanism of ciliary beating direction in *Paramecium*.

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